HISTONE PROPINQUITY USING IMIDOESTERS

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SUMMARY: Native chromatin was reacted with bifunctional protein cross-linking reagents of varying molecular sizes. Short cross-linkers do not produce significant yields of polymeric histones whereas intermediate-sized materials such as dimethyladipimidate produce extensive interaction between histones. Dimethylsuberimidate reacts with all histone fractions but only generates polymers of F_1 and of F_{2b} and F_{2a2}.

Great interest is currently centered upon chromatin structure and upon the organization of histones upon DNA (1-3). Isolated histone fractions interact together strongly (4,5) and dimethylsuberimidate has been utilized to cross-link the components of such histone oligomers (6). We were curious therefore, to see if similar cross-linked products can be obtained from native chromatin, thus providing information about the way in which histones are organized on the DNA molecule. This report describes the use of three bifunctional imidoesters to this end and compares the results with those obtained recently utilizing glutaraldehyde (7, 8).

MATERIALS AND METHODS

Chromatin was isolated as described previously (9). It was adjusted to a concentration of 500 μg/ml DNA and 5×10^{-4} M triethanolamine hydrochloride pH 7.4. Solutions of the imidoesters were prepared immediately before use at 20 mg/ml in the same buffer and the pH adjusted to 7.4 using NaOH. The final concentration of imidoester in the chromatin solution was 1 mg/ml. After fixation for a given time at 4° the reaction mixture was adjusted to 0.4 N H_2SO_4 and kept in ice for 60 min. The material was centrifuged at 14,000 rpm/90 min in a Sorvall RC-2B centrifuge. The supernatant was dialyzed against ethanol to precipitate histones and polymer histones. Subsequent

analysis was on polyacrylamide gels (10). Gels were scanned in a Beckman Acta III gel scanner.

Suberimidate-fixed histones were partially fractionated using the methods of Johns (11). F_1 was extracted in 5% perchloric acid; F_{2a} and F_3 were extracted in 80% ethanol - 0.25 N HCl, leaving F_{2b} insoluble.

RESULTS AND DISCUSSION

The time course for fixation of chromatin by dimethylsuberimidate is shown in Fig. 1. The band patterns in acid-urea gels become somewhat blurred as a function of time, no doubt due to the uptake of the cross-linking reagent. There is a rapid decrease in the amount of F_1 histone present, this is followed by a steady decrease in the amount of monomer F_{2a2} and F_{2b}. Concurrently we

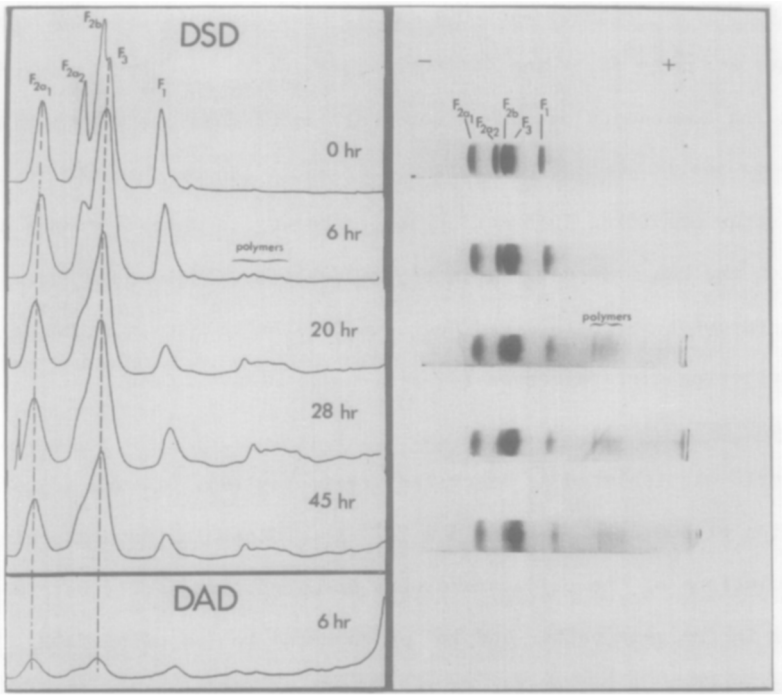

Figure 1. Fixation of histones by dimethylsuberimidate on native chromatin. At times indicated the cross-linking reaction was terminated and histones were isolated and analyzed in the acid-urea electrophoretic system (10). The gels (right) show unpolymerized histone as a function of time, together with polymer material migrating primarily in the position normally occupied by dimers. Microdensitometer scans of the gels appear at left with dimethyladipimidate fixation after 6 hrs shown for comparison in the bottom panel. The direction of migration is from right to left.

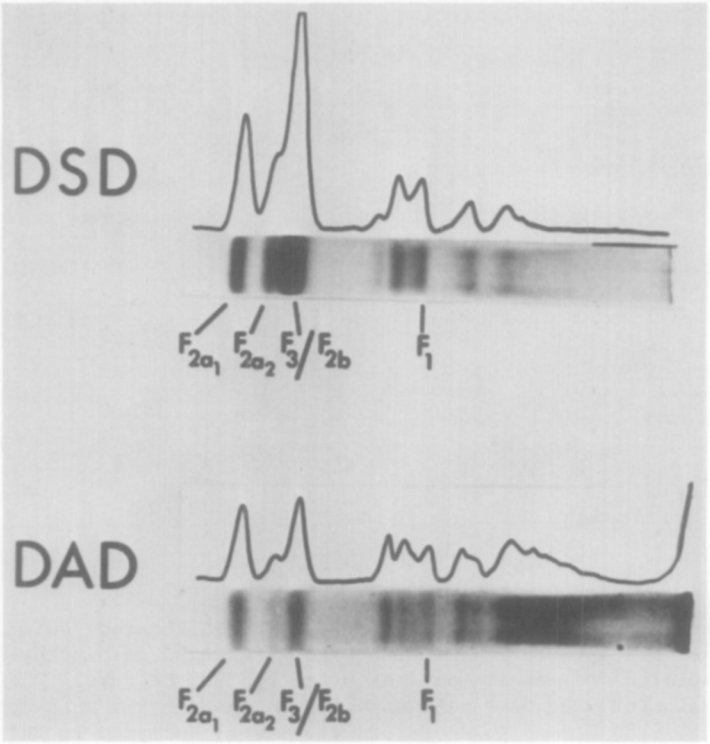

Figure 2. SDS-gel electrophoresis of 28 hr suberimidate-fixed (DSD) and 6 hr adipimidate-fixed (DAD) chromatin. Histone and oligomers were extracted as described in the text, dissolved in SDS-buffer at pH 10.5 and analyzed in the SDS electrophoretic system (10).

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see the appearance of material moving with about 50% of the mobility of monomer histones and at later stages considerably higher molecular weight material is produced. The material with mobility 50% of that of the monomer histone moves in the same region as F_3 dimers and it seems reasonable to ascribe it to various dimers of the histones which are present in ever decreasing amount as the fixation continues. As this material does not build up, it seems likely that it is converted in part to higher polymers as the reaction continues. The gel patterns of Fig. 1 indicate that after 20 hrs a limit is reached and

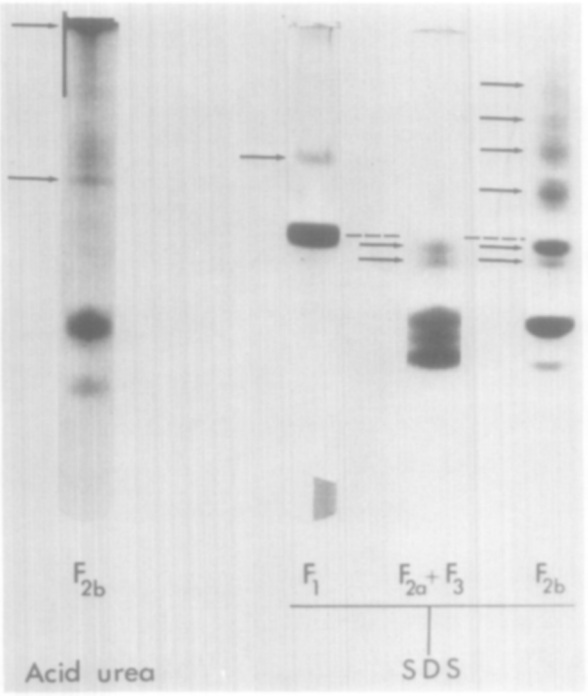

Figure 3. Electrophoretic analysis of partially fractionated, suberimidate-fixed histones. Histones in native chromatin were fixed in dimethylsuberimidate for 20 hrs, isolated and fractionated as described in the text. The F_{2b} sample (contaminated with 5% F_{2a1}) was analyzed in both the acid-urea and SDS gel systems. The F_1 and $F_{2a} + F_3$ fractions were analyzed in the SDS system. Oligomer bands are denoted with arrows. F_1 in SDS gels migrates more slowly than the dimer bands, as indicated by the dotted line.

this is shown clearly on the microdensitometer traces. During the first 28 hrs of the reaction about 70% of the F_1, F_{2b} and F_{2a2} was polymerized and is not present in the monomer region, whereas F_{2a1} and F_3 appear to have undergone relatively little cross-linking reaction. When the reaction was continued for 45 hrs relatively little change was noted, though a small fraction of F_{2a1} and F_3 has been removed at this time. The products from the polymerization of F_{2b}, F_1 and F_{2a2} may be noted as slower migrating fractions on these scans. This material is highly complex and as many as 6-7 bands may be detected.

Utilizing dimethyladipimidate instead of the suberimidate reveals a remarkable difference. All histone fractions can be cross-linked and disappear

from the gel, though the order of reaction is similar to that seen for the suberimidate. A typical residual pattern after 6 hrs of treatment is shown in Fig. 1. In contrast dimethylmalonimidate generates no detectable yield of histone polymers and histone monomers do not disappear from the gel even after extended times of reaction.

The products from the suberimidate and adipimidate fixations were analyzed on SDS gels and the results are shown in Fig. 2. The gel patterns of the suberimidate reaction are particularly interesting. Only three classes of polymer are seen, which by analogy with our recent work on glutaraldehyde fixation we would classify as dimers, trimers and tetramers. The relative proportions are indicated in the scans of Fig. 2. Extraction of F_1 reveals that it may be contributing to the slower-moving band, but otherwise the F_1 appears to be lost as much higher molecular weight material which does not enter the gel, and thus we conclude that much of the polymeric material has its origin in F_{2b} and F_{2a2} histones. The gel pattern for adipimidate fixation is somewhat similar to that for the suberimidate-treated material except for two critical differences. These are (1) many higher molecular weight polymers are now observed so that a continuum of material is seen above the level of the intermediate oligomers, and (2) the complexity of the different levels of oligomer is greater than seen following suberimidate fixation.

The limited polymer production after incubation with suberimidate is not due to hydrolysis of imidoester, as addition of extra material during the course of reaction did not change the time course of polymerization.

I have utilized the chemical fractionation techniques of Johns (11) to further probe the nature of the polymers produced by reaction of chromatin with dimethylsuberimidate. The analysis of the products is shown in Fig. 3 and it is apparent that a minor contribution to one of the higher level polymers is coming from F_1, that the bulk of the polymers is found in the F_{2b} fraction and that the entire F_{2a} and F_3 fraction contains very little polymeric material.

Reaction of chromatin at pH 7.4 with dimethyladipimidate leads to complete

polymerization of histones so that after several hours they can no longer be extracted into dilute acid. Since material which is acid-soluble during the course of the reaction contains complex histone polymers, we may conclude that histones are cross-linked together and that either the very high molecular weight polymers become insoluble in acid, or that they also become cross-linked to non-histone proteins or DNA.

Since the reaction of chromatin with dimethylsuberimidate follows a simple course, this system may prove much more amenable to detailed chemical analysis of histone propinquity than adipimidate or glutaraldehyde. Only three of the five histones are polymerized by this agent, about 75% of each of these three fractions (F_1, F_{2b} and F_{2a2}) are involved in cross-linking reactions at a stage when essentially none of F_3 or F_{2a1} have become cross-linked. The products for this reagent are mostly small oligomers of F_{2a2} and F_{2b} and higher molecular weight polymers of F_1 which eventually become insoluble in acid. The tendency to form fairly large polymers of F_1 now appears to be well documented (7, 8). If the oligomers of F_{2b} and F_{2a2} are analyzed on a logarithmic scale, they appear to be in groups differing by approximately 14,000 daltons, and we suspect that they are dimers, trimers and tetramers. There is apparently relatively little homopolymer of the type $(F_{2a2})_n$ as the polymers tend to separate into the F_{2b} fraction upon chemical separation. Thus we incline to the notion that the oligomers are either a mixture of homopolymers of F_{2b} and heteropolymers of F_{2b} and F_{2a2} or consist primarily of the latter possibility. Obviously F_{2a1} and F_3 are able to react with imidoesters since they are able to form cross-links either to themselves or to other histone fractions using adipimidate (or glutaraldehyde) and we may conclude that the lysine residues in these arginine-rich histones involved in adipimidate cross-links are situated in close proximity and the suberimidate is simply too large a molecule for correct orientation for reaction. [It is intriguing to note that isolated, free F_{2a1} and F_3 are readily polymerized by dimethylsuberimidate to give mixtures of small oligomers (6).] Thus the arginine-rich his-

tones can become involved in cross-links (either to themselves or to other histones) if the cross-linker is longer than 6 Å (malonimidate does not form cross-links) or shorter than 13.5 Å (suberimidate). Within this range we find cross-linking by adipimidate (10.5 Å) and glutaraldehyde (9 Å). Consistent with this proposal are the recent observations that we obtain no fixation with 4,4'-difluoro-3,3'-dinitrodiphenyl sulfone (14.6 Å) or glyoxal (4.5 Å).

Cross-linking is the ultimate result of a reaction sequence with two different rate constants. On the one hand there is the initial attack of the cross-linking reagent to a lysine residue to form a monofunctional imidoester and this is followed by the attack of the imidoester upon a lysine residue in a nearby histone. If this lysine is appropriately situated, one would expect the second reaction to proceed rapidly. Presumably the reason F_{2a1} and F_3 are not converted into histone polymers at the same rate as F_{2a2} and F_{2b} is because the lysine residues are not appropriately organized and the second reaction is much slower than the first and most of the available lysine residues become bound to monofunctional imidoesters so that ultimately few free amino groups are available for cross-linking. However repeating the suberimidate reaction at a much lower input concentration of di-imidoester did not change the relative rates of reaction, nor did the relative amounts of the F_{2a2}, F_{2b} oligomers change significantly.

Since it is generally acknowledged that almost all of the DNA is associated with histone, it follows that the distribution of F_1 in sequences and of F_{2b} and F_{2a2} in such a manner that the major products are small oligomers imposes certain constraints upon the organization of F_3 and F_{2a1}, particularly since the total amount of F_{2b} and F_{2a2} quite closely approximates that of F_3 and F_{2a1}. It would seem to be unlikely that all the F_3 and F_{2a1} are exclusively in the form of tetramers (or higher oligomers) nor can they exist entirely separate from each other (i.e. interspersed with other histones such as F_{2b} F_3 F_{2a2}). Thus we may anticipate that F_3 and F_{2a1} are mostly organized in rather small groups of several of 1, 2, 3 or 4 histones.

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